

# Effects of chronic ozone exposure on gene expression in *Arabidopsis thaliana* ecotypes and in *Thellungiella halophila*

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## ABSTRACT

*Arabidopsis thaliana* (At) ecotypes Columbia-0 (Col-0), Wassilewskija (WS), Cape Verde Islands (Cvi-0) and a relative, *Thellungiella halophila* (Th), were exposed to 20–25% over ambient ozone [O<sub>3</sub>] in a free air concentration enrichment (FACE) experiment (<http://www.soyFACE.uiuc.edu>), mirroring increases expected in the near future. Col-0 and WS accelerated development and developed lesions within 10 d under increased ozone, while Cvi-0 and Th grew slowly. RNAs were used in microarray hybridizations (Col-0-based 26 000 elements, 70-mer oligonucleotides). A two-step analysis of variance (ANOVA) model, including comparison with values obtained under [O<sub>3</sub>], was used for analyses. WS showed the greatest number of changes in gene expression in response to ozone. Th showed the least changes, suggesting that its expression state at [O<sub>3</sub>] was sufficient for resistance at increased ozone. Patterns observed in ambient air controls for Cvi-0 and Col-0 were most similar, while Th showed the greatest number of differences compared with the other controls. Compared with Col-0, however, Cvi-0 showed higher levels of expression of chaperones, receptor kinase-like and photosynthesis-related genes in ambient air. Cvi-0 exhibited ozone-mediated changes in a pathway involving AtSR, a homologue of the mammalian NFκB family of redox-sensitive transcription factors, changes in chaperones, WRKY and C2H2 proteins and antioxidants. WS displayed ozone-mediated decreases in the expression of two AtSR/NFκB family members, C2-domain proteins and genes associated with cell wall growth and changes in the expression of marker genes for programmed cell death (PCD), among them *RCD1*, a key regulator in this pathway. Microarray data were verified by reverse transcriptase (RT)-PCR. We relate O<sub>3</sub>-response diversity across the four lines to different responses among signaling and transcriptional response networks and differences in gene expression at [O<sub>3</sub>] levels.

**Key-words:** FACE; quantitative PCR; ROS; stress acclimation; transcript profile.

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## INTRODUCTION

Daily, seasonal and manmade changes in tropospheric ozone concentrations can affect plant growth and can influence the composition of ecosystems (Heck *et al.* 1983; Pell, Schlaghauer & Arteca 1997; Vahala *et al.* 2003). At present, average regional concentrations of [O<sub>3</sub>] are 60 ppb. or exceeding this value, representing an increase from a preindustrial concentration of 10 ppb (Prather *et al.* 2003). In agricultural systems, different crop species react differently to increases in [O<sub>3</sub>], but current amounts and the 1–2% increase per year projected for the next 50 years will affect sensitive species, among them soybeans (Morgan, Ainsworth & Long 2003; Morgan *et al.* 2004). Recent intensive studies in controlled environments of ozone–plant interactions have yielded clues about molecular responses of leaf cells to challenges of ozone exposure (Rao & Davis 1999; Rao *et al.* 2000; Rao, Lee & Davis 2002; Overmyer, Brosche & Kangasjarvi 2003; Ahlfors *et al.* 2004; Booker *et al.* 2004; Tuominen *et al.* 2004). Ozone levels at, or considerably above, the highest (acute) levels normally occurring in the atmosphere have been used in these studies, and the controls used have been exposed to ozone-free air in controlled environment chambers. Entering through stomata, ozone dissolves in the apoplast, generating superoxide and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which then results in the production of additional reactive oxygen species (ROS) in oxidative bursts, most probably through the action of apoplastic NADPH oxidase (Laloi, Apel & Danon 2004). These ROS act as signaling molecules in redox-responsive pathways, which are only partially understood in any organism (Vranova, Inze & Van Breusegem 2002). Recognition of the redox state of proteins and membranes and consequent signaling to the genetic machinery have been reported from bacterial to mammalian species (Zheng, Aslund & Storz 1998; Nishi *et al.* 2002; Veal *et al.* 2003; Rodrigues-Pousada *et al.* 2004).

Acute ozone exposure results in the activation of a programmed cell death (PCD) response, very similar to the hypersensitive response that characterizes an incompatible plant–pathogen interaction (Kangasjarvi *et al.* 1994; Kangasjarvi, Jaspars & Kollist 2005; Overmyer *et al.* 2005). Ozone-induced PCD is characterized by an oxidative burst

and by the coincident induction of pathogenesis-related (PR) proteins (Rao & Davis 2001; Langebartels *et al.* 2002) as well as by antioxidant defense genes, such as *GSTI* and various ascorbate peroxidases (*APXs*) (Tamaoki *et al.* 2003). The participation of several plant hormones in response to acute ozone exposure, downstream of ROS production, has been established. Jasmonic acid (JA) appears to ameliorate ozone-induced PCD (Rao *et al.* 2000), while both salicylic acid (SA) and ethylene (ET) appear to propagate the damaging effect of high ozone levels (300 ppb, 4 h) (Rao *et al.* 2002). Vranova *et al.* (2002) outlined the complex plant hormones–ROS interactions, in which ROS may act as secondary messengers, and pointed to upstream reactions involving ROS that precede the participation of hormones. Rao & Davis (1999) and Rao *et al.* (2000) compared acute ozone responses by two *Arabidopsis thaliana* (*At*) ecotypes, Cape Verde Islands (*Cvi-0*) and Columbia (*Col-0*). *Cvi-0* proved more sensitive to 300 ppb ozone for 3 h than *Col-0*, with ozone-free air acting as a control. We distinguished between the induction of defense processes by ROS and a less sensitive ROS-mediated pathway(s), involving SA and leading to PCD, and attribute the greater sensitivity of *Cvi-0* to activation of the PCD pathway. The results also suggest an antagonistic relationship between SA and ET, promoting PCD and JA, lessening the incidence of PCD (Tuominen *et al.* 2004). Several studies have traced the effects of ozone on photosynthesis, including reports using the SoyFACE (soy free air concentration enrichment) system (Oksanen, Sober & Karnosky 2001; Wustman *et al.* 2001; Morgan *et al.* 2004). Antioxidant detoxification systems have been identified as key players in resistance to ozone (Conklin & Last 1995; Pitcher & Zilinskas 1996).

Here we report the behavior of *At* ecotypes, *Cvi-0*, Wassilewskija (*WS*), and *Col-0*, and the *At* relative, *Thellungiella halophila* (*Th*), grown under field conditions in moderately elevated ozone for 10–14 d in FACE (free air concentration enrichment) rings. Continued development in ambient (*c.* 60–90 ppb) and elevated ( $1.2 \times$  ambient)  $O_3$  rings characterized *Cvi-0* and *Th*, whereas *Col-0* and, in particular, *WS* suffered injury under the same exposure conditions. The relative resistances of *Col-0* and *Cvi-0* were reversed under the lower ozone levels used under FACE conditions, compared with results reported for acute, high-concentration exposures. A gene expression study determined possible bases for this result, i.e. whether the same or different pathways responded to low ozone levels.

The gene expression profiles for *WS* suggest that the ozone level used may have been sufficient to trigger hormone-related pathways leading to PCD, while *Col-0*, *Cvi-0* and *Th* showed significantly more resistance, i.e. different pathways may have been triggered in the different ecotypes and in *Th* by low ozone levels. Additional comparisons between ambient air samples of the *At* ecotypes and *Th* revealed a possible correlation between stress-resistance capacity and the level of 'constitutive' expression of stress-related transcripts as has previously been

proposed (Donahue *et al.* 1997; Inan *et al.* 2004; Taji *et al.* 2004). Expresso analyses of the data resulted in the identification of pathways that seem to distinguish among ecotypes. Expresso performs statistical analyses of raw spot intensity data using a two-stage, global, mixed-linear model, adapted from the model in Wolfinger *et al.* (2001), that identifies effects from multiple factors (array, printing pin, dye, spot position, block and treatment). The Expresso analysis is run on the statistical analysis software (*SAS*) platform and returns both a differential change for each gene under each treatment and a statistical significance (*P*-value) for that change. Smallfold yet statistically significant changes can be identified and can be made available for further analysis (Jin *et al.* 2001; Stasolla *et al.* 2003; Watkinson *et al.* 2003).

We propose the engagement of a novel stress-resistance pathway in *Cvi-0* in the field at  $1.2 \times [O_3]$ , compared with ambient open-air ozone levels, that may be independent of the less sensitive, established, hormone-related pathway leading to PCD documented to operate at much higher ozone levels under controlled laboratory conditions.

## MATERIALS AND METHODS

### Plant material and $O_3$ exposure

Seeds of three *At* ecotypes (*WS*, *Col-0* and *Cvi-0*) and of *Th*, ecotype Shandong (Bressan *et al.* 2001), were placed on trays containing soil from SoyFACE (<http://www.soyFACE.uiuc.edu>). After vernalization (4 °C, 3–4 d), trays were moved into a greenhouse at ~25 °C and 600–700  $\mu\text{moles photons m}^{-2} \text{s}^{-1}$  until the plants had reached the rosette stage and then were transplanted into SoyFACE. We used three control rings (ambient air) and three rings in which  $[O_3]$  was elevated from the ambient concentration to 20–25% above ambient (Miglietta *et al.* 2001; Morgan *et al.* 2004). The three locations served as biological repeats. Variables describing weather conditions were recorded at 10 min intervals and may be obtained at <http://www.soyface.uiuc.edu/weather/July2003.xls>. Plants were grown in full sunlight in an area cleared of soybean plants within the rings. Watering was done in the early morning as needed. Within days after transplanting, foliar disease symptoms appeared, manifested by chlorophyll loss in small areas or entire leaves and as localized necrotic lesions. Ecotype *WS* was most susceptible to damage, while *Cvi-0* was largely unaffected.

Plants were harvested after growth in the field for 8–12 d. Material sampling was determined by visual inspection, omitting plants with damaged or senescing leaves. At least 10 plants per experiment were sampled from each FACE ring. Plants were cut at ground level, immediately frozen in liquid  $N_2$  and stored at –80 °C. For control purposes, RNAs isolated from plants from two different rings for the same treatment were labelled with cy3 and cy5 fluorescent dyes, respectively, and hybridized against each other. Correlation in these control hybridizations ranged between 0.90 and 0.97 (Miyazaki *et al.* 2004).

## Physiological parameters

Two leaves from at least three plants of each line were selected at 2 day intervals during the 12 d the plants stayed in the rings with measurements carried out at midday. The measurements posed considerable problems because of leaf size and damage. Leaf CO<sub>2</sub> uptake ( $A$ ), stomatal conductance to water vapour ( $g_s$ ) and intercellular CO<sub>2</sub> concentration ( $C_i$ ) were measured in an open gas exchange system with an integrated fluorescence chamber head using a leaf cuvette designed for *At* leaves (LI-6400-40, Li-Cor, Lincoln, NE), where the [CO<sub>2</sub>] in the cuvette was set to that of ambient air for the given plot. Responses of  $A$  to  $C_i$  were measured and  $V_{c,max}$  and  $J_{max}$  were calculated (Bernacchi *et al.* 2003). Apparently healthy plants were selected, with most rosette leaves lacking insect or obvious fungal and/or bacterial damage, although leaf colouration increased in [O<sub>3</sub>] rings. No distinction was made with respect to border effects, because plants used were spatially separated. All ecotypes and *Th* showed stomatal conductance to water vapour ( $g_s$ ) in elevated [O<sub>3</sub>] 20–30% lower compared to plants in control rings. Damage of leaves generated considerable variability of the data and prevented a more in-depth analysis.

## RNA extraction and microarray hybridization

Total RNA was obtained by using a method developed for tissues with high carbohydrate content (Jaakola *et al.* 2001). RNA was further purified using RNeasy columns (Qiagen, Carlsberg, CA, USA). Total RNA, 100 µg per sample, was labelled using the indirect labeling procedure as described by Hedge *et al.* (2000). RNA was mixed with 0.8 mM of dATP, dCTP and dGTP; 0.5 mM of dTTP and 0.3 mM of 5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate (Sigma, St Louis, MO, USA); 2 µg oligo dT (Invitrogen, Carlsbad, CA, USA) followed by first-strand cDNA synthesis (Superscript II RT; Invitrogen). After incubation for 2 h at 42 °C, the cDNA was treated with 2 units of RNaseH (Invitrogen) for 15 min at 37 °C and purified (Qiagen). After binding the cDNA, the columns were washed with phosphate-ethanol buffer (5 mM potassium phosphate, pH 8.0 and 80% ethanol), and the cDNA was eluted with phosphate buffer (4 mM K-phosphate, pH 8.5).

The purified cDNA was dried before the coupling reaction with Cy3-dUTP or Cy5-dUTP dye ester (Amersham-Pharmacia, St Louis, MO) in 0.1 M Na<sub>2</sub>CO<sub>3</sub> (pH 9.0) and the labelled cDNA was purified (Qiagen). Cy3- and Cy5-labelled cDNAs were dissolved in hybridization buffer [25% formamide, 5 (SSC), 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT) and 100 µg/mL sonicated salmon sperm DNA) and combined. Glass slides were pretreated and hybridized (Kawasaki *et al.* 2001). Hybridizations were done after the competitive two-dye (Cy3 and Cy5) method. RNA extracted from control rings was used as the counterpart for RNA from [O<sub>3</sub>] rings. In total, three hybridizations were done, with dye swaps.

Microarray slides including >26 000 elements consisting of 70-mer gene-specific oligonucleotides were used (see: <http://www.ag.arizona.edu/microarray/microarray/>). After hybridization, the slides were scanned and spot intensities were extracted (Genepix 4000 scanner, GenePix Pro 3.0, Molecular Devices Corp., Sunnyvale, CA, USA). Three replicates were used for the comparison of results of elevated [O<sub>3</sub>] versus control rings.

## Quantitative reverse transcriptase (RT)-PCR

Two micrograms of total RNA was incubated at 50 °C (2 h) with 0.5 µg of Oligo-OligodT dT primer (Invitrogen), 0.5 mM dNTP, 5 mM DTT and 1 µL of Superscript-III TM-RNaseH minus RT (Invitrogen) in 20 µL of 1 × reaction buffer supplied by the manufacturer. Real-time PCR was performed in a total volume of 25 µL using 2 µL of the 20 times diluted first-strand cDNA synthesis mixture as a template ('reaction buffer'), 100 PM forward primer, 100 PM reverse primer and 12.5 µL of 2 × SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Primers for PCR were designed using Gene Runner and were purchased from Invitrogen. The nucleotide sequences of the primers were checked for their specificity in the *The Arabidopsis* Information Resource (TAIR) Basic Local Alignment Search Tool (BLAST) database. Actin-3 (forward, 5'-CAAAGGCTAACCGTGAGAAGATGACTC-3'; reverse, 5'-GACCACTAGCATAAAGCGACAGGACAG-3') was used as an internal control. The following genes were chosen: *At1g05010* (*EFE*), *At1g11910* (*Asp* protease), *At1g32230* (*CEO1*), *At1g32330* (*HSF8*), *At1g68550* (*AP2*), *At1g72050* (*C2H2* zinc), *At1g72260* (*THI2.1*), *At1g77020* (*HSP*), *At2g14610* (*PRI*), *At2g22300* (*NFκB* homologue 2), *At2g28190* (*CSD2*), *At2g37040* (*PAL1*), *At2g38920* (*C3HC4* RING finger), *At2g41460* (*APR*), *At2g45050* (*GATA* zinc), *At2g46680* (*AtHB7*), *At3g04120* (*GAPC*), *At3g22360* (*AOX1b*), *At3g44880* (*ACD1*), *At3g45440* (receptor-like protein kinase), *At3g47480* (calcium-binding EF-hand family protein), *At4g11650* (*ATOSM34*), *At4g26080* (*ABII*), *At4g31940* (*P450*), *At5g23310* (*FSD3*), *At5g44420* (*PDF1.2*) and *At5g45890* (*SAG12*). Each sample was analysed in triplicate. The universal thermal cycling parameters were used as recommended (10 min activation at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C). Experiments were carried out and results were calculated using the ABI PRISM 7700 (Foster City, CA, USA) sequence detection system. Primer sequences and results are deposited in Supplemental Data S1a and S2.

## Data analysis

Analyses used Expresso, a system of tools and databases for integration of computation and experimentation in the context of gene expression experiments (Watkinson *et al.* 2003). The slide design, with various controls in each block, suggests the use of a statistical model using the controls. Results were stored in a relational database with relevant metadata.

The sensitivity of individual genes to the experimental treatments was estimated using a two-stage statistical analysis (Wolfinger *et al.* 2001), including a term for an effect that evaluated the blocks on the microarray slides. The inclusion of all spot measurements, including control clones and clones without At numbers, improved the estimation of the variance of the block effect. These clones were included in the normalization model as 'genes' but were labelled as such to distinguish them from the experimental genes.

The two-stage analysis was implemented using SAS (SAS/STAT Software version 8.2, SAS Institute Inc., Cary, NC). The least square means of gene treatment interactions were estimated. For each gene, the significance of the differences in least square means between the stress level and control were estimated using Tukey-Kramer multiple comparison procedure. Each difference constitutes the estimated log<sub>2</sub>-fold change for that gene. A gene with positive (respectively, negative) estimated fold change at  $\alpha=0.01$  was marked positively (respectively, negatively) expressed. Remaining genes for which no change was observed are marked unchanged. Two treatments (stressed and control) compared each of the At ecotypes, Col-0, Cvi-0, WS and Th.

## RESULTS

### Responses to elevated ozone

We compared RNAs of plants from [O<sub>3</sub>] and ambient air rings (<http://soyFACE.uiuc.edu>) and analysed their transcript profiles using whole genome, long-longoligonucleotide microarray slides. RNAs were from At ecotypes Col-0, WS and Cvi-0 and from the At relative, Th, harvested after 8–12 d growth under field conditions. Cvi-0 performed best and showed no lesions, possibly indicating successful acclimation, while WS developed lesions, often showed strong pigmentation, and lower stomatal conductance to water vapour (*g*<sub>s</sub>). The progressive decrease over time of available large and undamaged leaves, however, precluded gathering data suitable for a sound statistical analysis. Col-0 and, to a lesser extent, WS, immediately

initiated flowering; Cvi-0 did not. Th showed little or no damage but grew more slowly than any of the At ecotypes.

### Gene expression analysis

All data are the result of the statistical analysis and are listed in Supplementary Table S3. The model (Wolfinger *et al.* 2001) was modified to accommodate the block design of the microarrays used. The numbers of genes responding across all comparisons and in all lines after analysis are shown (Table 1 and Supplemental Table S1b). We compared expression patterns among controls and also examined the stress-control contrast within ecotype or species. Among the lines, WS showed more down-regulated genes than the other ecotypes or Th, and Th showed the lowest number of genes responding to ozone, 140+ and 187–. In contrast, Cvi-0 showed a relatively large number of down-regulated genes (735). Among the control comparisons, the Th/WS contrast showed the greatest number of down-regulated genes, where 4177 genes were more highly expressed in WS ambient air controls than in Th controls. The values obtained from comparing the different controls with each other are considerably higher than those obtained from comparing stressed with control treatments for Th, Col-0 and Cvi-0, except for the Col-0/Cvi-0 comparison. WS, however, showed a total of 2926 genes that responded to the ozone stress. Under the conditions in the field, the expression state of each ecotype in ambient air differs from that of the others, with the greatest contrast between Th and WS (6081), and the smallest contrast between Col-0 and Cvi-0 (1091). A comparison of increased ozone versus ambient control in WS may yield important information concerning pathways deleteriously affected by exposure to low ozone levels, while other comparisons may give clues on gene expression patterns associated with adaptive responses to [O<sub>3</sub>]. A cross-ecotype comparison of stress-associated expression patterns is likely to have the most meaning for Cvi-0 and Col-0. A comparison of ambient control patterns between Th and WS may lead to a better understanding of expression states associated with adaptation (Th) versus injury (WS).

**Table 1.** Comparisons of numbers of genes differentially expressed in Col-0, Cvi-0, WS and Th in response to ozone and among their respective controls

	Col-0 (O <sub>3</sub> ) versus Col-0 (co)	Cvi-0 (O <sub>3</sub> ) versus Cvi-0 (co)	Th (O <sub>3</sub> ) versus Th (co)	WS (O <sub>3</sub> ) versus WS (co)	Col-0 (co) versus Cvi-0 (co)	Col-0 (co) versus Th (co)	Col-0 (co) versus WS (co)	Cvi-0 (co) versus Th (co)	Cvi-0 (co) versus WS (co)	Th (co) versus WS (co)
Positive (+)	410	568	140	802	649	2379	732	2482	919	1904
Negative (-)	220	735	187	2124	442	2529	2875	2536	2839	4177
No change (0)	21 903	21 110	21 924	19 848	21 388	17 038	18 902	16 889	18 716	16 202
No <i>P</i> -value	208	120	186	390	56	74	206	69	192	126

The columns indicate positive (+), negative (-) or unchanged (0) expression. 'X' indicates that the *P*-value was not estimated by statistical analysis software (SAS) PROC MIXED, therefore the level of expression is not assessed.

Col-0, Columbia-0; Cvi-0, Cape Verde Islands; WS, Wassilewskija; Th, *Thellungiella halophila*; (O<sub>3</sub>), free air concentration enrichment (FACE) ozone rings; (co), FACE control rings.

Data displayed in Fig. 1 show stress-induced changes in gene expression for core metabolism in Th and WS (Fig. 1a & b) and a comparison between Cvi-0 and WS stress (Fig. 1c), while additional comparisons are provided as Supplementary Fig. F1a–d.

WS showed the greatest number of changes in core metabolism in response to the increase in [O<sub>3</sub>]. Relative to ambient air, genes associated with the photosynthetic machinery were down-regulated in WS, as were transcripts for enzymes in sucrose biosynthesis, the ascorbate-glutathione scavenging cycle and cell wall metabolism genes. In contrast, core metabolism genes were little affected by exposure to ozone in Cvi-0, Col-0 or Th (Supplementary Data S4a, b and c). In Fig. 1c, the results of the

comparison of the core metabolism gene expression in the Cvi-0 and the WS stressed samples are shown. Genes associated with the light reactions were more highly expressed in Cvi-0 than in WS, whereas the genes of the flavonoid and phenylpropanoid pathways, likely indicators of perceived stress, showed increased expression in WS. Light reaction genes, the reductive pentose phosphate pathway (RPPP), scavenging pathway and photorespiration were also more highly expressed in Cvi-0 than in Th (Supplementary Data S4a, b and c). A comparable contrasting pattern is seen for the Col-0/Cvi-0 ambient control for the light reactions and the RPPP, where Cvi-0 shows higher expression of photosynthesis-related genes than Col-0. Table 2 lists all regulated transcripts in chloroplast-related categories

**Table 2.** Effect of exposure to ozone on photosynthesis-related genes. Differential expression of Col-0, Cvi-0, TH and WS and comparisons of Col-0 and Cvi-0 controls

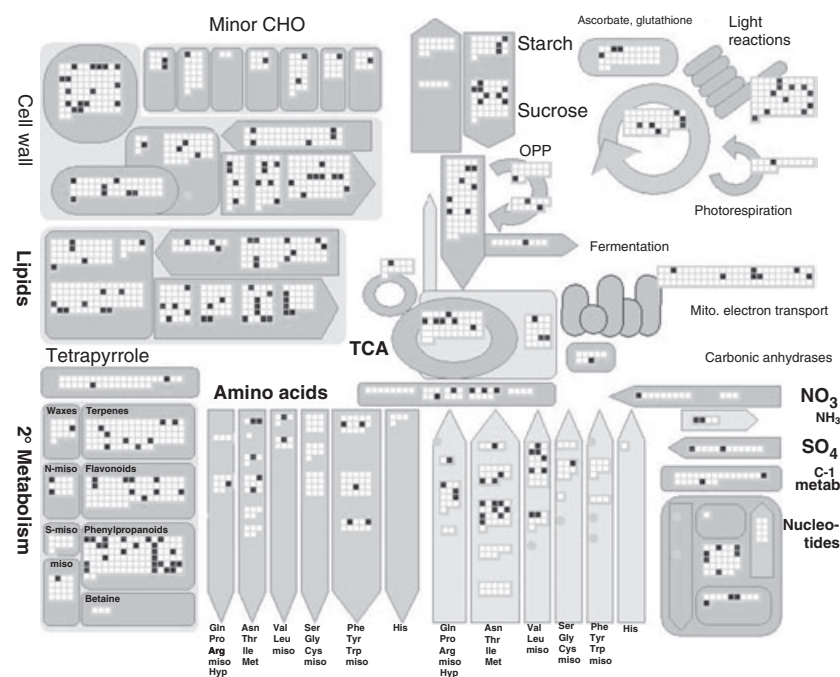
Gene	Annotation	Col-0 (stress) versus Col-0 (control)	Cvi-0 (stress) versus Cvi-0 (control)	TH (stress) versus TH (control)	WS (stress) versus WS (control)	Col-0 (control) versus Cvi-0 (control)
<b>Thylakoid associated</b>						
<i>AT1G09750</i>	chloroplast nucleotide DNA-binding protein-related	0	0	0	–	–
<i>AT2G28800</i>	chloroplast membrane protein (ALBINO <sub>3</sub> , ALB3, OXA1p)	0	0	0	0	–
<i>AT5G15350</i>	plastocyanin-like domain containing protein	0	0	0	–	–
<i>AT5G53490</i>	thylakoid luminal 17.4 kDa pentapeptide repeat family protein, chloroplast precursor	0	0	0	0	–
<i>AT1G12250</i>	chloroplast lumen pentapeptide protein, putative	0	0	0	0	–
<i>AT3G52380</i>	chloroplast RNA-binding protein cp33	0	0	0	0	–
<i>AT1G55670</i>	photosystem I subunit V precursor, putative	0	0	0	0	–
<i>AT2G39470</i>	oxygen-evolving complex 25.6 kDa protein, chloroplast precursor, putative	0	0	0	0	–
<i>AT3G56650</i>	thylakoid luminal 20 kDa protein	0	0	0	0	–
<i>AT1G14150</i>	PsbQ domain protein family	0	0	0	0	–
<i>AT3G63490</i>	chloroplast ribosomal L1 – like protein	0	0	0	–	–
<i>AT2G44920</i>	thylakoid lumen pentapeptide repeat family protein	0	0	0	0	–
<i>AT1G30380</i>	photosystem I subunit X precursor	0	0	0	0	–
<i>AT1G54780</i>	thylakoid lumen 18.3 kDa protein	0	0	0	0	–
<i>AT5G20720</i>	chloroplast Cpn21 protein	–	–	0	–	–
<i>AT5G02590</i>	chloroplast lumen common protein family	0	–	0	–	–
<i>AT2G34420</i>	photosystem II type I chlorophyll <i>a/b</i> binding protein	0	+	0	0	–
<b>Stroma associated</b>						
<i>AT3G01850</i>	putative D-ribulose-5-phosphate 3-epimerase	0	0	0	–	0
<i>AT1G43670</i>	fructose 1,6-bisphosphatase, putative	0	0	0	0	–
<i>AT3G55800</i>	sedoheptulose-bisphosphatase precursor	0	0	0	0	–
<i>AT1G63290</i>	putative D-ribulose-5-phosphate-3-epimerase	–	0	X	0	0
<i>AT1G17745</i>	D-3-phosphoglycerate dehydrogenase (3-PGDH)	+	–	+	–	+
<i>AT3G14930</i>	uroporphyrinogen decarboxylase, putative	0	–	0	–	–
<i>AT5G40850</i>	uroporphyrin III methylase (gb1/2AAB92676.1)	0	0	0	0	–
<i>AT1G01080</i>	chloroplast RNA-binding protein cp33, putative	–	0	X	0	–
<i>AT1G52560</i>	chloroplast-localized small heat shock protein, putative	0	0	0	0	+
<i>AT3G18630</i>	uracil-DNA glycosylase, putative	0	0	–	0	0
<i>AT4G25120</i>	UvrD/REP helicase family protein contains Pfam PF00580: UvrD/REP helicase	0	0	–	X	0

Genes in the category carbon metabolism showed differential expression (Wolfinger *et al.* 2001). Expression is indicated as positive (+), negative (–) or unchanged (0). ‘X’ indicates that the *P*-value could not be calculated. Cells with solid borders indicate the genes up-regulated in the Cvi-0 control and may predispose the ecotype for acclimation. Cells bordered by broken lines indicate genes that responded in Col-0, and cells bordered by double line indicate genes that responded in WS.

Col-0, Columbia-0; Cvi-0, Cape Verde Islands; Th, *Thellungiella halophila*; WS, Wassilewskija.

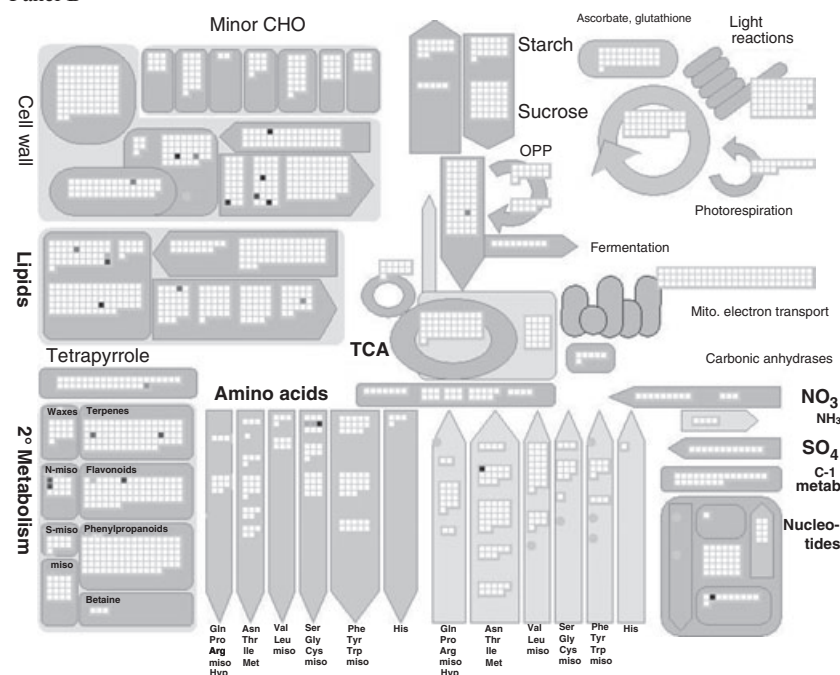
(a)

Panel A



(b)

Panel B



**Figure 1.** Effect of ozone exposure on gene expression of metabolism in different ecotypes. Gene expression changes are depicted in MapMan format (Thimm *et al.* 2004). The complete data set used for MapMan analysis is listed in Supplemental Data S3. (a) Panel A: effect of ozone exposure on Wassilewskija (WS). (b) Panel B: effect of ozone exposure on *Thellungiella halophila* (Th). (c) Panel C: a comparison of the core metabolism gene expression in Cape Verde Islands (Cvi-0) and WS. MapMan is a user-driven tool that paints gene expression data sets onto diagrams of metabolic pathways or other processes (Thimm *et al.* 2004). Each square represents a gene. Red, blue and white meant up-expression, down-expression and no change in expression, respectively. Gradient of the colours depict the strength of gene expression. Supplementary Tables 4a–c contain the annotation of the genes highlighted in panels A, B or C, respectively.

identifying those that showed statistically significant differences. A group of photosynthesis-related genes are more highly expressed in Cvi-0 than in other ecotypes, regardless of the presence of the stress condition (Supplemental Data S5).

### Antioxidant functions

The responses of antioxidant genes to ozone and across the controls are shown in Table 3. In the scavenging cycle, the thylakoid-bound ascorbate peroxidase (tAPX) expression

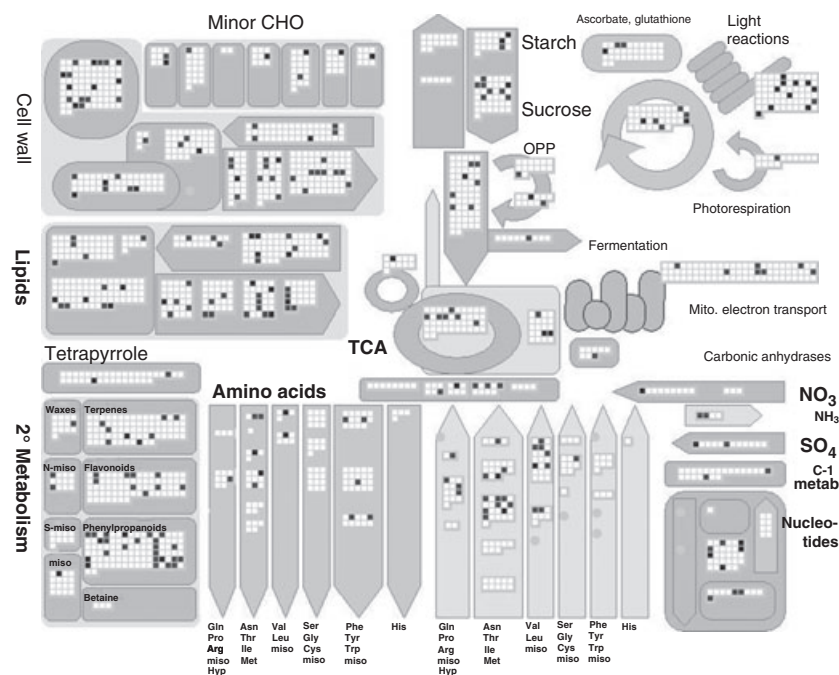
(c)  
Panel C

Figure 1. Continued

**Table 3.** Effect of ozone exposure on antioxidant genes. Differential expression of Col-0, Cvi-0, TH and WS and comparisons of Col-0 and Cvi-0 controls

Gene	Annotation	Col-0 (stress) versus Col-0 (control)	Cvi-0 (stress) versus Cvi-0 (control)	TH (stress) versus TH (control)	WS (stress) versus WS (control)	Col-0 (control) versus Cvi-0 (control)
Scavenging cycle						
<i>AT1G07890</i>	APX, putative	0	0	0	0	0
<i>AT1G77490</i>	tAPX, putative	0	0	0	-	-
<i>AT3G09640</i>	APX	-	0	0	0	+
<i>AT2G28190</i>	CSD2	-	0	0	0	-
<i>AT1G12520</i>	chaperone, putative		0	0	0	0
<i>AT3G10920</i>	Mn-SOD1	0	0	0	-	0
Glutaredoxins						
<i>AT3G62930</i>	glutaredoxin-like protein	0	+	0	0	0
<i>AT1G03850</i>	glutaredoxin protein family glutaredoxin family protein contains Pfam profile PF00462: glutaredoxin	0	+	0	0	0
<i>AT3G15660</i>	glutaredoxin-like protein	0	0	0	-	0
<i>AT3G62950</i>	glutaredoxin-like protein	0	0	0	-	0
Thioredoxins						
<i>AT1G50950</i>	thioredoxin	0	0	0	-	0
<i>AT5G39950</i>	thioredoxin	0	0	0	-	0
<i>AT4G27080</i>	thioredoxin	0	0	0	-	0
<i>AT1G53300</i>	thioredoxin	0	0	0	+	0
<i>AT2G35010</i>	thioredoxin	0	0	0	+	0
<i>AT2G41680</i>	thioredoxin	0	0	0	0	-

Antioxidant genes are classified into functional groups. Changes in transcript profile are shown as positive (+), negative (-) or unchanged (0). 'X' indicates that the *P*-value could not be calculated. Cells with solid borders indicate the genes up-regulated in the Cvi-0 control and may predispose the ecotype for acclimation. Cells bordered by broken lines indicate genes that responded in Col-0, and cells bordered by double line indicate genes that responded in WS.

Col-0, Columbia-0; Cvi-0, Cape Verde Islands; Th, *Thellungiella halophila*; WS, Wassilewskija; APX, ascorbate peroxidase; tAPX, thylakoid-bound ascorbate peroxidase; CSD2, copper/zinc superoxide dismutase; Mn-SOD1, manganese superoxide dismutase.

was depressed by exposure to ozone in WS, while it was more highly expressed in Cvi-0 than in either Th or Col-0. This pattern of an O<sub>3</sub>-dependent decrease in WS was also observed for stromal Cu/Zn superoxide dismutase 2, and for mitochondrial manganese superoxide dismutase (Mn-SOD1). Seemingly, the photosynthetic machinery might be protected more against oxidative stress in Cvi-0 than in the other lines or in Th. No ozone-mediated changes in scavenging genes were observed in Th.

Two glutaredoxin genes were more highly expressed in Cvi-0 in ozone compared with WS, Col-0 and Th while, in contrast to all other lines, two different glutaredoxins showed lower expression in WS. Hypothetically, the Cvi-0 glutaredoxins that responded to ozone could contribute to the superior resistance of Cvi-0 to ozone compared with Col-0. Under control conditions, Cvi-0 and Col-0 showed equal expression of all glutaredoxin genes. No glutaredoxin gene responded to ozone in Th. Genes encoding two different glutaredoxins were expressed at a higher level in Th than in Col-0 or WS controls. Thioredoxin genes that responded showed a distinct pattern (Table 3). No response to ozone was recorded for Col-0, Cvi-0 or Th, while WS showed a number of thioredoxin transcripts declining and two increasing. The Col-0/Cvi-0 comparison showed four thioredoxin genes more highly expressed in Cvi-0 than in Col-0 controls.

### Hormone-related genes

A number of genes in functions related to ET, JA and SA biosyntheses and action responded to chronic ozone exposure with responses that seem to indicate ecotype-specific sensitivity differences.

#### ET

Cvi-0 showed increased expression of three known ET-responsive genes with unknown functions under ozone (Table 4). The expression of *At5g44440*, *At2g26020* and *PDF1.2a/b* were decreased under ozone exposure in both Cvi-0 and Col-0. ET-responsive genes did not respond to ozone exposure in Th and neither did ET biosynthesis genes, whereas three *ACC* oxidase genes responded positively in WS and one in Cvi-0. The expression of one *ACC* synthase *AtACS-6* (*At4g11280*) was depressed by exposure to ozone in WS only. Ozone exposure resulted in increased expression of *PR-4* (*At3g04720*) in WS. Decreases were seen in ozone-exposed plants for three *NFκB*-like genes 1, 3 and 5 (*At5g09410*, *At5g64220* and *At3g16940*) in WS and an increase in another *NFκB*-like gene (#2), *At2g22300* in Cvi-0.

#### JA

Table 4 also shows data obtained for JA biosynthesis genes and for genes or their close relatives known to be JA responsive. JA biosynthesis does not appear to have responded to ozone exposure in Cvi-0 and Th, while in Col-

0, a lipoxygenase gene, *At1g72520*, showed lower expression.

#### SA

SA is a component in the pathway leading to ozone-mediated PCD, with gene *PR1* (*At2g14610*) being a key player. Expression of *PR1* was increased by exposure to ozone in Cvi-0 from a control level of expression that was already higher than in Col-0. The transcript encoding a PR5-like protein (*At1g19320*) increased in Col-0.

### Transcription factors

Significantly regulated transcripts in this category included *WRKY*, *C2H2*, *C3HC4* and *C2* domain genes (Supplementary Data S8). In ambient air, we observed no differences between Col-0 and Cvi-0 for either *C2H2* or *C2* domain containing proteins, and no effect of ozone on gene expression was found in Col-0, Cvi-0 or Th. Exposure to ozone resulted in higher expression of two *C2H2*-type genes in Cvi-0, which was contrasted by reduced expression of eight *C2* domain genes in WS. Three genes in the *WRKY* family increased in expression with ozone. One of these, *At5g01900*, has been reported to participate in SA-triggered defense gene expression through activation of *NPR1*, an *IKB* homologue (Yu, Chen & Chen 2001). No *WRKY* gene was more highly expressed in Cvi-0 than in Col-0 controls. Two *HSFs* were expressed under ozone exposure in Cvi-0 [*HSA1d* (*At1g32330*) and *HSA7b* (*At3g63350*)]. Components of a proposed ROS signaling pathway [the Zn-finger protein *Zat12* (*At3g18290*) and *WRKY25* (*At2g30250*)] did not show changes in expression in response to ozone in either of the lines/ecotypes (Rizhsky *et al.* 2004).

### Arabinogalactans (AGPs), receptor-like kinases (RLKS), mitogen-activated protein kinases (MAPKS) and the cell wall-membrane continuum

This group is represented by genes with significant changes under control conditions among the ecotypes and Th and by changes in expression in ozone (Supplementary Data S9). The majority of the RLKS that showed differences in gene expression in the comparisons are associated with the endomembrane system. Of 11 RLKS whose expression decreased upon exposure to ozone in WS, only *TMK1* (*At1g66160*) is an extracellular kinase and may therefore be a participant in an ozone-responsive signaling pathway originating in the apoplast. The expression of nucleus-located *ATR1* (*At5g60890*) decreased in the presence of ozone in Col-0. Three endomembrane RLKS (*At3g14640*, *At4g04570* and *At5g01550*) were expressed at a higher level in Cvi-0 than in either Col-0 or Th. The expression of three FLA-type AGPs (*FLA 2*, *8* and *10*) decreased under ozone in WS. Differences were only apparent among MAP kinases that have not been previously related to ozone

**Table 4.** Effect of ozone exposure on hormone-associated genes. Differential expression of Col-0, Cvi-0, TH and WS and comparisons of Col-0 and Cvi-0 controls

Gene	Annotation	Comments	Col-0 (stress) versus Col-0 (control)	Cvi-0 (stress) versus Cvi-0 (control)	TH (stress) versus TH (control)	WS (stress) versus WS (control)	Col-0 (control) versus Cvi-0 (control)
ET responsive							
<i>AT1G04370</i>	ET response factor, putative		0	0	0	X	X
<i>AT1G49830</i>	ET-responsive protein		0	+	0	0	-
<i>AT1G50640</i>	ET-responsive element binding factor 3 (AtERF3)		0	0	0	0	-
<i>AT1G53170</i>	ET-responsive element binding factor 8		0	0	0	0	-
<i>AT1G55150</i>	ET-responsive DEAD/DEAH box RNA helicase protein, putative		0	+	0	0	0
<i>AT5G44210</i>	ET-responsive element binding factor 9 ET-insensitive 3 family protein contains Pfam profile: PF04873		0	0	0	-	0
<i>AT5G65100</i>	ET-insensitive 3		0	0	0	-	0
<i>AT3G04720</i>	hevein-like protein precursor (PR-4)	1	0	0	0	+	0
<i>AT2G22300</i>	ET-induced calmodulin-binding plant defensin protein, putative	NGκB2-F	0	+	0	0	0
<i>AT5G44420</i>	(PDF1.2a) plant defensin protein, putative	1	-	-	0	0	0
<i>AT2G26020</i>	(PDF1.2b)	1	-	-	0	0	0
1 = Marker for ozone-mediated ET induction							
ET biosynthesis							
<i>AT1G05010</i>	1-aminocyclopropane-1-carboxylate oxidase		-	0	0	+	0
<i>AT2G19590</i>	1-aminocyclopropane-1-carboxylate oxidase		0	0	0	0	-
<i>AT5G43440</i>	1-aminocyclopropane-1-carboxylate oxidase		0	+	0	0	+
<i>AT5G43450</i>	1-aminocyclopropane-1-carboxylate oxidase		0	0	0	0	0
<i>AT5G59530</i>	ACC oxidase-like protein		0	0	0	+	0
<i>AT5G59540</i>	ACC oxidase-like protein		0	0	0	+	0
<i>AT1G77330</i>	ACC oxidase, putative		0	0	0	0	0
<i>AT3G61400</i>	ACC oxidase-like protein		0	0	0	0	+
<i>AT1G01480</i>	1-aminocyclopropane-1-carboxylate synthase (ACC synthase)		0	0	0	0	0
<i>AT3G47190</i>	1-aminocyclopropane-1-carboxylic acid oxidase-like protein		+	0	0	0	0
<i>AT5G63600</i>	1-aminocyclopropane-1-carboxylic acid oxidase-like protein		0	0	0	0	+
<i>AT4G11280</i>	ACC synthase (AtACS-6)		0	0	0	-	0
JA induced							
<i>AT4G24190</i>	HSP90-like protein	cell rescue/defense		0	0	0	-
<i>AT2G24850</i>	putative tyrosine aminotransferase	3 protein	-	-	0	0	+
<i>AT4G01310</i>	L5 ribosomal protein	synthesis		0	0	0	-
<i>AT5G57090</i>	auxin transport protein EIR1 (gb1/2AAC39513.1)	2	0	0	0	-	0
<i>AT5G24780</i>	vegetative storage protein Vsp1	3	0	0	0	0	+
<i>AT5G24770</i>	vegetative storage protein Vsp2	2	0	0	0	0	+
JA biosynthesis							
<i>AT1G17420</i>	lipoxygenase	LOX3	0	0	0	+	0
<i>AT1G55020</i>	lipoxygenase		0	0	0	0	+
<i>AT1G72520</i>	lipoxygenase		-	0	0	+	+
2 = marker for ozone-mediated JA induction; 3 = JA-inducible							
SA induced							
<i>AT2G14610</i>	PR-1-like protein	4	0	+	0	0	-
<i>AT1G19320</i>	pathogenesis-related protein 5 precursor, putative	4	+	0	0	0	+
4 = Marker for ozone-mediated SA induction							
<i>RCD1/CEO</i>							
<i>AT1G32230</i>	WWE domain-containing protein/CEO protein, putative (CEO)		0	0	0	-	0

Table 4. Continued

Gene	Annotation	Comments	Col-0	Cvi-0	TH	WS	Col-0
			(stress)	(stress)	(stress)	(stress)	(control)
			versus	versus	versus	versus	versus
			Col-0	Cvi-0	TH	WS	Cvi-0
			(control)	(control)	(control)	(control)	(control)
<i>AT2G35510</i>	WWE domain-containing protein NFκB and related genes		0	0	0	–	0
<i>At5g09410</i>	calmodulin-binding protein; protein	NFκB1	0	0	0	–	0
<i>AT2G22300</i>	ET-induced calmodulin-binding	NFκB2-F	0	+	0	0	0
<i>AT5G64220</i>	calmodulin-binding protein	NFκB3-F	0	0	0	–	0
<i>AT1g67310</i>	calmodulin-binding protein	NFκB4	0	0	0	0	0
<i>AT3G16940</i>	calmodulin-binding protein	NFκB5	0	0	0	–	0
<i>AT3G59700</i>	S/T-specific kinase lecRK1	IKK4-F	0	0	0	+	0

Hormone-associated genes are grouped by a hormone or a signaling pathway. Changes in transcript profile are shown as positive (+), negative (–) or unchanged (0). 'X' indicates that the *P*-value could not be calculated. Cells with solid borders indicate the genes up-regulated in the Cvi-0 control and may predispose the ecotype for acclimation. Cells bordered by broken lines indicate genes that responded in Col-0, and cells bordered by double line indicate genes that responded in WS.

Col-0, Columbia-0; Cvi-0, Cape Verde Islands; Th, *Thellungiella halophila*; WS, Wassilewskija; JA, jasmonic acid; SA, salicylic acid; ET, ethylene; PR, pathogenesis-related.

responses. AtMPK5, MAPKK2 and MAPKK4 declined in ozone in WS, while AtMPK7 increased in Th (Supplementary Data S6).

### Senescence

Ecotype WS, in particular, showed signs of senescence. This ecotype also showed responses of known senescence-related transcripts in ozone (Supplemental Data S7). These included a number of functionally unknown senescence-indicator transcripts, and transcripts encoding patatin-like or LIM proteins, and a cyclic nucleotide-regulated ion channel. Other ecotypes showed fewer responses among senescence-related protein transcripts.

### Control analyses by quantitative RT-PCR

To assess the reliability of results from microarray expression profiles of FACE-grown plants, real-time PCR analyses were included with cDNAs synthesized from the RNAs used for array hybridization. All analysed genes exhibited apparent up- or down-regulation in real-time PCR experiments that correlated with the microarray results with a correlation coefficient ranging from 0.63 to 0.92 in different lines (Supplemental Data S2), indicating overall reliability of the microarray results.

## DISCUSSION

The availability of the SoyFACE facility during the soybean growing season provided an opportunity to analyse effects of chronic ozone exposure on At ecotypes (Col-0, Cvi-0 and WS) and the At relative Th, which is characterized by extreme tolerance to several abiotic stresses (Bressan *et al.* 2001). While FACE facilities provide realistic growth conditions, it must be understood that any results obtained

depend not only on local weather conditions at the time of growth and harvesting, but also on responses to biotic factors (in our case, pathogen and insect attack) during the exposure period. Results from FACE experiments – while they are consistent within one set of conditions, with plants in blocks grown at the same location and time – will not be comparable with plants grown at different times. Ozone- and ROS-related effects have recently been analysed in controlled environments, generally in short-term, acute exposure experiments (Overmyer *et al.* 2000, 2005; Ahlfors *et al.* 2004; Tuominen *et al.* 2004). Ecotype differences with respect to acute ozone exposure have been reported (Overmyer *et al.* 2000; Baluska *et al.* 2003; Johnson *et al.* 2003; Sivaguru *et al.* 2003; Tamaoki *et al.* 2003; Ahlfors *et al.* 2004; Kacperska 2004). Results from these experiments implicate an involvement of multiple factors that contribute to ozone sensitivity.

Our objectives were twofold. Firstly, responses of At ecotypes have been analysed at the transcript level in controlled environments under acute ozone treatments, with ozone-free air as the control. Thus, it seemed of interest to relate the results from these studies to results obtained from chronic exposure under field conditions (ambient versus 1.2 × ambient). The field conditions used here mirror predicted changes in atmospheric ozone in the future (Morgan *et al.* 2004). Transcriptome status in Col-0 in ambient air in the FACE system has already been reported substantially different from comparable data obtained from plants grown in controlled, ozone-free environments (Miyazaki *et al.* 2004). In the FACE system, transcriptome responses reflect any differing behavior in the relatively subtle range of approximately 20 ppb higher than the ozone concentration normally experienced by the plants in a daily range from 40 to 100 ppb, depending on local weather conditions. Contrasting the different strategies is also interesting in light of data on aspen ozone responses

(Vahala *et al.* 2003), suggesting that distinct ozone-responsive mechanisms may operate depending on the level of ozone and on acute or chronic stress. Our data support such a hypothesis.

Secondly, the microarray tools available for *At*, which have been used for its close relative *Th* as well (Inan *et al.* 2004; Taji *et al.* 2004), could provide additional information about ecotype or species differences through transcript profiles. If this could be demonstrated, FACE experiments might become a vehicle for comparisons between (breeding) lines and species under natural conditions.

Discovery of an ozone-sensitive mutant, *rcd1* (Col-0), showed the *RCD1* gene to be a modulator of responses to ET, methyl jasmonate and abscisic acid (ABA), conferring resistance specifically to apoplastic ROS (Overmyer *et al.* 2000, 2005; Ahlfors *et al.* 2004), the initial site of ozone action. Putative sensors at the cell wall–plasma membrane interface, such as the AGPs and wall-associated receptor kinases, are candidates for participation in *RCD1*-mediated pathways (Baluska *et al.* 2003; Johnson *et al.* 2003; Sivaguru *et al.* 2003; Kacperska 2004). *At CEO1* (*RCD1*) complemented an oxidative stress-sensitive yeast mutant (Belles-Boix *et al.* 2000). *RCD1* could be a part of a signaling pathway common to stresses involving ROS, activating ROS-resistance mechanisms.

### The basis for ozone susceptibility in WS

Countering ozone-mediated effects leading to PCD, JA is thought to protect against ET- and SA-triggered events (Overmyer *et al.* 2003). The data support the view that WS may be enhancing JA biosynthesis and JA-inducible genes under ozone exposure, based on up-regulation of *Lox3* and *PDF1.2*, which are JA-inducible (Lorenzo *et al.* 2001; Spoel *et al.* 2003). JA's role is seen in lesion containment that purportedly follows injury of cell membranes (Overmyer *et al.* 2005). Apparently, ozone injury exceeded a threshold leading to induction of JA-related processes in WS but not in the other lines. We see further evidence for such a process in the behavior of *RCD1/CEO1* and the closely related *SROI* (Ahlfors *et al.* 2004), which are both negatively regulated in WS without a corresponding response in the other lines. Conceivably, the ozone level used in the rings failed to trigger this resistance pathway in Col-0, Cvi-0 and in *Th*.

Although JA has long been viewed as ameliorating effects of ET- and SA-mediated damage by ozone, WS suffered the greatest degree of injury. This may be due to the decreased expression of *RCD1* in WS and of other genes participating in signaling pathways in the cell-wall plasma membrane continuum. Acute exposure leads to progressive lesion formation in *At* Col-0 *rcd1* (Ahlfors *et al.* 2004), providing evidence for a protective function for this pathway. The *RCD1* protein modulates ABA, ET and methyl JA responses (Ahlfors *et al.* 2004; Overmyer *et al.* 2005). The down-regulation of this pathway in WS may cause ozone sensitivity, although we saw no evidence in the data for activation of ET or SA biosynthesis in WS. The

expression of markers for activation of the PCD pathway, including *PR-4*, was also increased in ozone-exposed WS, mirroring the phenotypic appearance.

### Redox-mediated signaling via *NFκB* homologues

Yang & Poovaiah (2002) showed differential responses to JA, ET, H<sub>2</sub>O<sub>2</sub> and extremes of temperature among members of the calmodulin-binding protein family AtSR1-6 (CAMTA). AtSR family members show homology to redox-responsive mammalian NFκB transcription factors, whose movement from the cytosol to the nucleus is controlled by cellular redox state (Kabe *et al.* 2005). Ozone-mediated down-regulation of NFκB-1, -3 and -5 homologues was observed only in WS. Bouche *et al.* (2002) presented evidence for a nuclear location of AtSR proteins and delineated the DNA-binding capacity of two conserved protein domains. *AtSR* genes, such as the genes that responded in WS in this study, could therefore participate in ozone-responsive signaling pathways that initiate defense gene induction and/or PCD, depending on the level of stress imposed. The AtSR/NFκB homologue proteins are candidates for redox sensing that may act together with, or parallel to, the *RCD1* pathway.

### Extracellular site of ozone action

No evidence from the expression profiles of any of the four lines is found for an involvement of the proposed participants in the *APX1/Zat12* pathway inferred by Davletova *et al.* 2005) and Rizhsky *et al.* (2004). This signaling pathway has, however, been linked to light stress acting on chloroplasts. Events connecting the action of *RCD1* (NFκB-like proteins, isoforms 3 and 5) with its site of action in the apoplast have still to be identified, although possible participants are suggested from our data from the down-regulation in WS of cell wall AGPs and TMK1, a cell wall-associated RLK. Two AGPs, FLA-2 and FLA-8, whose expression decreased in WS under ozone, are induced by ABA, and are therefore likely stress-responsive transcripts (Johnson *et al.* 2003).

### Map kinases

MAPK5 expression decreased under ozone in WS. Several reports implicate other MAPKs (*At*MAPK-3 and -6) in signaling in response to both biotic and abiotic stresses (Kovtun *et al.* 2000; Samuel, Miles & Ellis 2000; Bent 2001; Morris 2001; Moon *et al.* 2003), but little information is available on MAPK5. Grant, Yun & Loake (2000) demonstrated an additional H<sub>2</sub>O<sub>2</sub>-responsive pathway in *At* involving MAPKs, independent of ET, JA or SA under conditions that initiated HR and replicated acute ozone exposure. Our data suggests the participation of MAPK5 in a pathway adversely affected by chronic ozone exposure, at least in WSby.

## Gene expression patterns and the basis for the superior resistance of Cvi-0

### Redox sensing

The response of ecotype Cvi-0 to low-level ozone may be compared with that of WS and Col-0 with respect to several genes and their associated pathways. *AtSRI*, a homologue of the redox-sensing NFκB-2, is up-regulated under ozone in Cvi-0. Yang & Poovaiah (2002) reported that this gene is ET and JA responsive, but insensitive to H<sub>2</sub>O<sub>2</sub>. This opens up the possibility that the form of ROS underlying the increase in the expression of *AtSRI* is not H<sub>2</sub>O<sub>2</sub> but superoxide. It is less clear whether the level of ozone used in the field significantly affected ET biosynthesis, because the expression of ACC synthase, a key regulatory step in ET biosynthesis (Wang, Li & Ecker 2002), did not increase under ozone.

### Possible role of sa-triggered responses at chronic ozone levels

Accumulation of SA in high ozone is known to lead to systemic acquired resistance (Sharma & Davis 1994), and Cvi-0, at such an ozone stress, is more sensitive than Col-0 and accumulates SA (Rao *et al.* 2002). Rao & Davis (1999) reported that a relatively low threshold of SA induced by ozone is required for induction of antioxidant defense against the oxidative burst induced by the same signal, and a higher level of SA is required to initiate the PCD pathway. Indeed, *PRI*, a marker for ozone-mediated SA induction, is up-regulated in Cvi-0 in FACE. A possible contributing reason for the greater resistance of Cvi-0 to ozone compared with Col-0 in the FACE system is that the photosynthetic machinery appears to operate at a higher rate in Cvi-0 than in the other lines and in Th (Table 2). Cvi-0 appears better protected against ROS, as shown by the high gene expressions associated with chloroplast functions, including antioxidant genes. However, *APX1* (*Atlg07890*), a key gene in the ROS-responsive pathway (Davletova *et al.* 2005), showed no differences. Considering that the *APX1* pathway is associated with events initiated in the chloroplast, the results further support an initial site of ozone action in the apoplast. The differences in mechanism that we infer from our data conform to a view that, at low (ambient) O<sub>3</sub>, Cvi-0 is better protected against injury than Col-0. This may be due to an adaptive response on the part of Cvi-0 to [O<sub>3</sub>] levels. At the high (acute) O<sub>3</sub> stress levels that have been used in other studies, when SA levels in Cvi-0 are excessive, cell death may be activated.

Cvi-0 was unique in showing higher expression of three *WRKY* family genes in ozone. One of these (*At5g01900*) participates in SA-triggered defense gene expression through activation of *NPRI*, an *IKB* homologue (Yu *et al.* 2001). This finding seems to support our hypothesis and the assumption that SA levels increased with chronic ozone exposure in Cvi-0 to levels sufficiently high to trigger a defense pathway, but not so high as to lead to PCD, which

is in agreement with the phenotypic appearance of the plants.

From chamber studies, Rao *et al.* (2000) reported Cvi-0 as less JA-responsive than Col-0. In high ozone, more pronounced injury resulted in Cvi-0, correlated with higher levels of SA and with lower levels of JA responses in Cvi-0. Under our conditions, the less pronounced sensitivity of Cvi-0 to induce the JA pathway, as reflected also in our data, had no effect. This reversal of relative sensitivities under chronic as opposed to acute ozone stress, resembles observations made with hybrid aspen genotypes (Vahala *et al.* 2003).

## The basis for ozone sensitivity in Col-0 in FACE

Compared with Cvi-0 and Th, Col-0 showed injury, albeit at a lower degree than WS. Its gene expression profile, however, showed no evidence for a hormone-related PCD pathway or the putatively more responsive resistance pathway seen in Cvi-0.

Interpreting the ecotype-specific expression profiles, we infer the existence of a novel stress-resistance pathway in Cvi-0 that may be independent of the less sensitive, established hormone-related pathway to PCD documented to operate at high ozone. One contributing factor may be higher expression, at [O<sub>3</sub>] levels, of key stress-related genes in the more readily adaptive ecotypes, compared with WS and Col-0 that exhibited injury. The NFκB2 homologue and/or an *HSF* homologous to mammalian *HSF21* could be candidate redox sensors in Cvi-0, and two *WRKYs* may participate in the pathway.

FACE provided an excellent opportunity to gauge responses of the At ecotypes in a natural setting and the effects of chronic, low-level ozone exposure on the transcriptome, compared with the acute and brief exposures more commonly used. Not surprisingly, each ecotype (and Th) exhibited distinguishing characteristics, both in their stress-response kinetics and in the magnitude of the responses recorded by microarray hybridizations and phenotype. In addition, ecotype differences in the presence of ROS response chains in [O<sub>3</sub>] seem to predetermine successful adaptation to 1.2 × [O<sub>3</sub>] or to drive the plants to PCD or senescence. The differential sensitivities of the ecotypes and Th under chronic conditions that mirror future environmental conditions, as opposed to acute ozone exposure, observed in the FACE system, i.e. the apparent reversal of sensitivities of Col-0 and Cvi-0, constitute evidence for the existence of multiple ROS-sensing and resistance pathways, such as has been proposed by Munnik & Musgrave (2001) for drought-sensing mechanisms in At. These putative pathways may be distinguished both on the basis of the subcellular locations from where the ROS arise (e.g. the apoplast for ozone stress versus the chloroplast for light stress) and on the threshold of oxidative stress required to elicit a response from the transcriptome. A thorough comparison of circumstances leading to adaptation/resistance under controlled conditions which more closely emulate those occurring in the field, versus those leading to injury, may

yield more insights into the complex relationship between the behavior of multiple redox-sensing pathways and differential ROS-tolerance across the ecotypes.

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### Supplementary material

The following supplementary material is available for this article online:

**Figure S1.** A comparison of the expression of core metabolism genes in Cvi-O and WS under ozone stress.

**Table S1.** Primers for real time PCR.

**Table S2.** Correlation between quantitative RT-PCR and microarray measurements in different Arabidopsis ecotypes and *T. halophila*.

**Table S3.** Expressed genes,  $\alpha = 0.01$ .

**Table S4.** Core metabolism genes from Th that are shown in Map Man diagram.

**Table S5.** Differential expression of photosynthetic related genes.

**Table S6.** Differential expression of MAPKs.

**Table S7.** Differential expression of Senescence.

**Table S8.** Effect of ozone exposure on transcription factor genes.

**Table S9.** Effect of ozone exposure on receptor-like kinases and AGP genes.